Application of direct polymerase chain reaction assays for *Campylobacter fetus* subsp. *venerealis* and *Tritrichomonas foetus* to screen preputial samples from breeding bulls in cow-calf herds in western Canada

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Abstract

The primary objectives of this study were to estimate the prevalence of *Campylobacter fetus* subsp. *venerealis (Cfv)* and *Tritrichomonas foetus* in breeding bulls from a sentinel cohort of cow-calf herds in western Canada and to estimate the association between positive test status and non-pregnancy. The final objective was to evaluate the application of these tests when: i) screening bulls in the absence of a recognized problem with reproductive performance, and ii) testing for diagnosis of poor pregnancy rates. The crude apparent bull prevalence for *Cfv* was 1.1% [95% confidence interval (CI): 0.5% to 2.1%; 8/735] and herd prevalence was 2.6% (95% CI: 0.3% to 9.0%; 2/78). The crude apparent bull prevalence for *T. foetus* was < 0.001% (95% CI: 0.0% to 0.5%; 0/735) and herd prevalence was < 0.001% (95% CI: 0.0% to 4.6%; 0/78). Cows from herds where at least 1 bull was test positive for *Cfv* were 2.35 times more likely (95% CI: 1.01% to 5.48%; $P = 0.047$) to not be pregnant than those with no positive bulls. Polymerase chain reaction (PCR) testing of preputial material collected into phosphate-buffered saline (PBS) was recommended for screening for *T. foetus* when the pre-test probability of infection was > 1%. The same test for *Cfv* was not recommended for screening moderate- and low-risk herds due to the high risk of false positives. Tests for both *T. foetus* and *Cfv* can be used to investigate herds with reproductive problems when also ruling out other risk factors. Regardless of the type of test used, however, 3 negative tests are required to rule out infection in high-risk situations.

Résumé

Les objectifs primaires de la présente étude étaient d'estimer la prévalence de Campylobacter fetus *ssp.* venerealis (Cfv) *et* Tritrichomonas foetus *chez des taureaux reproducteurs d'une cohorte sentinelle issue de troupeaux vache-veau dans l'ouest canadien et d'estimer l'association entre un test positif et la non-gestation. L'objectif final était d'évaluer l'application de ces tests lors de : i) vérification des taureaux en absence d'un problème reconnu avec les performances de reproduction, et ii) épreuve diagnostique en présence de faibles taux de gestation. La prévalence apparente brute des taureaux pour* Cfv *était de 1,1 % [intervalle de confiance (IC) 95 % : 0,5 % à 2,1 %; 8/735] et la prévalence pour les troupeaux était de 2,6 % (IC 95 % : 0,3 % à 9,0 %; 2/78). La prévalence apparente brute des taureaux pour* T. foetus *était < 0,001 % (IC 95 % : 0,0 % à 0,5 %; 0/735) et la prévalence pour les troupeaux était < 0,001 % (IC 95 % 0,0 % à 4,6 %; 0/78). Les vaches provenant de troupeaux où au moins un taureau s'était avéré positif pour* Cfv *étaient 2,35 fois plus susceptibles (IC 95 % : 1,01 à 5,48;* P = *0,047) de ne pas être gestante que celles provenant de troupeaux sans aucun taureau positif. L'analyse par réaction d'amplification en chaine par la polymérase de matériel prépucial prélevé dans de la saline tamponnée était recommandée pour vérifier la présence de* T. foetus *lorsque la probabilité d'infection pré-test était > 1 %. Le même type d'analyse pour* Cfv *n'était pas recommandé pour la vérification des troupeaux à risque modéré et faible étant donné le risque élevé de faux positifs. Les tests pour* T. foetus *et* Cfv *peuvent être utilisés pour investiguer les troupeaux avec des problèmes de reproduction en même temps que les autres facteurs de risque sont éliminés. Toutefois, indépendamment du type de test utilisé trois tests négatifs sont requis pour éliminer la possibilité de l'infection dans les situations à risque élevé.*

(Traduit par Docteur Serge Messier)

Introduction

Campylobacter fetus subsp. *venerealis (Cfv)* and *Tritrichomonas foetus (T. foetus)* are important causes of reproductive loss in herds that use natural breeding. While individual case reports have documented costly herd outbreaks in western Canada (1), to date no studies have investigated the frequency or prevalence of *T. foetus* and *Cfv* infection in beef bulls and cow-calf herds in western Canada.

The clinical presentation of these venereal pathogens is very similar and both pathogens should be considered when investigating herds with poor fertility (2). Chronically infected bulls are an important reservoir in maintaining and transmitting both *T. foetus* and *Cfv.* A common option for controlling these infections is testing, identification, and culling of infected bulls (2).

While recent studies have measured the performance of diagnostic tests for *Cfv* and *T. foetus* (2–7), there are important differences in the

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availability, sensitivity, and specificity of tests for these 2 venereal pathogens. In addition to culture for *T. foetus*, which is available at many local clinics and laboratories, the use of polymerase chain reaction (PCR) tests for *T. foetus* is encouraged for both diagnosis and screening because of the reported improved sensitivity and specificity relative to culture (2,7,8). Culture of *Cfv* is more technically demanding, however, and is available only at some diagnostic laboratories. Culture of *Cfv* also has very low sensitivity when transport times to the lab exceed 24 h (2,9). Practical PCR tests for use on field samples for *Cfv* that require extended transport times have only recently become commercially available (3,5,10). The performance of PCR tests for *T. foetus* and *Cfv* differ (4–6), which raises questions about the relative interpretation and application of these tests in clinical practice.

The primary objectives of this study were to estimate the prevalence of *Cfv* and *T. foetus* in breeding bulls from a sentinel cohort of cow-calf herds in western Canada and to estimate the association between positive test status and the risk of non-pregnancy. The final objective was to evaluate various risk scenarios for the application of these tests when screening bulls: i) in the absence of a recognized problem with reproductive performance, and ii) when testing for diagnosis of poor pregnancy rates.

Materials and methods

In collaboration with local veterinarians across western Canada, cow-calf herd owners were recruited in 2014 and 2015 for a multi-year surveillance initiative. The herds were selected from various geographic regions in Alberta, Saskatchewan, and Manitoba to represent the distribution of herd sizes and density described in the 2011 Census of Agriculture data. Participants in the western Canadian Cow-Calf Surveillance Network were given the option to have their mature breeding bulls tested for infection with *Cfv* and *T. foetus* before the 2015 breeding season. Local veterinarians were paid up to $500 to cover additional costs associated with collecting and shipping samples from each herd. The costs for laboratory testing were also paid by the study.

Bull selection

Sampling for laboratory testing from mature bulls was offered to all participating herds as an extension of annual breeding soundness evaluations in the spring of 2015. Virgin bulls, either yearlings or virgin 2-year-olds, were not included in the study as infection with *T. foetus* or *Cfv* was considered to be very unlikely in these animals; therefore, the predictive value of a single positive sample would be very low as would the cost effectiveness of testing.

Sample collection

A single preputial sample was used to test for both *T. foetus* and *Cfv.* The preputial cavity was scraped repeatedly (at least 10 times) with an individually wrapped 50-cm AI pipette attached to a 20-cc syringe, with suction applied during scraping. The pipette was then flushed into a screw-top plastic vial containing 2.0 mL of phosphate-buffered saline (PBS) solution. As soon as possible after collection, the inoculated vial was placed in a refrigerator if the sample was shipped the same day or in a −20°C freezer if there was an expected delay in shipping. Samples were shipped in an insulated container with a frozen icepack to the Western College of Veterinary Medicine, Saskatoon, Saskatchewan.

Veterinarians were asked to complete a sample submission form requesting baseline information on each bull, including animal identification, age, and breed. Producers were also asked whether the bull was exposed to communal grazing in the previous breeding season and was semen tested by a veterinarian in 2015.

On arrival at the Western College of Veterinary Medicine, the preputial samples were thoroughly mixed and aliquoted for testing for *Cfv* within the research lab (KG, TW). The second aliquot was submitted to a commercial laboratory [Prairie Diagnostic Services (PDS), Saskatoon, Saskatchewan] for *T. foetus* testing. Submissions containing a positive *Cfv* sample based on results from the research laboratory were also submitted to the commercial laboratory (PDS) for second-laboratory confirmation using the same primer set described for the research laboratory.

Real-time PCR assay for *Cfv*

Deoxyribonucleic acid (DNA) was released from 200 μL of prepuce scrapings in PBS [20 mM phosphate, 150 mM sodium chloride (NaCl)] using a direct heat lysis with minor alterations (3,5). The preputial pellet was re-suspended in 100 μL of sterile water before heating, followed by a 1:10 dilution in sterile water before analysis (3,5). The real-time polymerase chain reaction (qPCR) mixture was created using SYBR green (iQ SYBR Green Supermix; Bio-Rad, Mississauga, Ontario), 400 nM of each primer, and 2 μL of dilute lysate in a final volume of 25 μL. All samples were run in duplicate on a thermocycler (iCycler/MyIQ; Bio-Rad) as previously described (3,5) using a primer set targeting *Cfv* (VenSF and VenSR) (11). Each test included no template and positive controls, also in duplicate. Melt-curve analysis was used to indicate infection status; the lower detection limit was 103 copies. The resulting data were analyzed using commercial software (iQ5 Optical System Software; Bio-Rad). Samples with a melt curvature signature comparable to the positive control, peak signal of 78.5°C ± 0.5°C [mean ± standard deviation (SD)], and threshold cycle *(Ct)* value of < 35 were considered positive.

The clinical sensitivity and specificity of the qPCR assay for *Cfv* has previously been reported for preputial samples collected directly into PBS (5). The crude sensitivity estimates from a group of 8 *Cfv*-positive bulls sampled repeatedly was 85.4% (95% CI: 80.6% to 89.2%; 222/260). Based on testing of 300 virgin bulls, the crude clinical specificity estimates for the qPCR assay were 85.0% (95% C: 80.5% to 88.6%; 255/300).

Real-time PCR for *T. foetus*

Direct preputial samples were tested with qPCR at a commercial diagnostic laboratory (Prairie Diagnostic Services, Saskatoon, Saskatchewan). DNA was extracted from a 200 mL aliquot of medium using a commercial kit (DNeasy Blood & Tissue BioSprint 96 One-For-All Vet Kit; Qiagen, Mississauga, Ontario). The qPCR assay was carried out using a Stratagene Mx3005P QPCR (Agilent Technologies Canada, Mississauga, Ontario), as described in a previous study (12). Samples were considered positive if the *Ct* value was < 40 (6).

Sensitivity of qPCR for *T. foetus* preputial samples collected directly into PBS was previously reported to be 90.1% (95% CI: 83.5%

Table I. Characteristics of the sample population (*n* = 735 bulls, N = 78 herds)

	Percent of bulls *(n)*	Percent of herds (N)	Median (IQR)	Range
Attributes				
Bull age (y)			4 (3, 5) (*n* = 690)	2 to 13 (*n* = 690)
Province				
Manitoba		19% (15)		
Saskatchewan		27% (21)		
Alberta		53% (41)		
British Columbia		1% (1)		
Breed				
Angus	27% (272)			
Simmental	22% (158)			
Charolais	9% (67)			
Gelbvieh	9% (66)			
Limousin	5% (39)			
Hereford	4% (32)			
Other	8% (64)			
Not reported	5% (39)			
Exposure to communal grazing (2013)	10% (73)	28% (22)		
Bulls semen tested by a veterinarian in current year (2015)	98% (717)	99% (77)		
Bulls semen tested by a veterinarian in previous year (2014)		90% (70)		
Bulls tested for *T. foetus* in previous year (2014)				
Yes		21% (16)		
Unsure		8% (6)		

IQR — Interquartile range.

to 94.2%; 109/121) for this laboratory (6). Although clinical specificity has not been reported for samples collected directly into PBS, the specificity of the assay from this laboratory for qPCR testing of samples collected in InPouch TF pouches (Biomed Diagnostics, San José, California, USA) was 100% (95% CI: 98.9% to 100%; 337/337) (4).

Reporting

The laboratory results for each herd were sent to the submitting veterinarian to share with the herd owner. Any herds that had a positive sample for *Cfv* or *T. foetus* were offered the choice of retesting their bulls.

A questionnaire was sent to producers in the fall of 2015 regarding conception outcomes for cows and heifers that were bred in 2014 to calve in 2015. Producers were asked to indicate how many cows and heifers were pregnancy checked and the number of open cows and heifers in the fall of 2014 or early winter of 2014 to 2015. Producers were asked to indicate if they only checked a portion of their animals, and if so, to estimate the open rate for both cows and heifers. Only the herds in which all cows and heifers were pregnancy tested were considered in the analysis of the association between infection status and pregnancy outcomes.

A questionnaire sent to all participants in the spring of 2014 provided baseline data on herd size and management. The requested information included whether a veterinarian had examined the bulls before the 2013 breeding season and whether the bulls were tested for *T. foetus* in the spring of 2013.

Statistical analysis

The participating herds and sampled bulls were described, as well as the pregnancy rates for the subset of herds in which all cows and heifers were pregnancy tested. A bull was considered test positive for either *Cfv* or *T. foetus* if the PCR test was positive at least once. A

Table II. Summary of available pregnancy test data for fall 2014 from 72 of the 78 herd owners responding to a survey and effect estimates for herd management factors that could be associated with risk of non-pregnancy

	Cows	Heifers
Herds that reported at least some pregnancy testing (N)	67	64
Median herd size (IQR)	200 (157 to 294)	40 (27 to 68)
Herds that reported pregnancy testing only a portion of the herd (N)	10	11
Herds that reported complete pregnancy testing (N)	57	53
Median herd size (IQR)	200 (159 to 300)	46 (30 to 68)
Risk of non-pregnancy		
Median (IQR)	6.5% (5.7% to 7.7%)	6.5% (3.3 to 14.7)
5th and 95th percentile	4.3%, 10.2%	0.0%, 22.5%
Potential risk factors for non-pregnancy other than exposure to *Cfv* and *T. foetus* (stratified by cows vs heifers) (n = 57)	RR (95% CI), P-value	
Exposure to communal grazing (yes/no)	0.81 (0.52 to 1.27), P = 0.36	
Herd size > 300 cows (yes/no)	0.82 (0.47 to 1.43), P = 0.48	
Time of bull exposure (before June/June or later)	1.82 (0.88 to 3.75), P = 0.11	
Calving started before March 2014 (yes/no)	1.38 (0.72 to 2.62), P = 0.33	
Calving season > 4 cycles or 84 d (yes/no)	1.13 (0.63 to 2.03), P = 0.67	
Sold purebred cattle (yes/no)	2.17 (0.89 to 5.32), P = 0.09	

IQR — Interquartile range; RR — Risk ratio; CI — Confidence interval.

herd was considered test positive if at least 2 bulls were test positive for *Cfv* or at least 1 bull was test positive for *T. foetus*. The difference in the number of reactors required for a herd to test positive was influenced by the lower specificity of the PCR test for *Cfv*.

The association between having at least 2 bulls that tested positive for *Cfv* or at least 1 bull that tested positive for *T. foetus* and the risk of non-pregnancy in the herds that pregnancy tested all of their females exposed to breeding was examined using generalized estimating equations. The data were examined using a negative binomial distribution with a log-link function and a robust variance estimate, while accounting for clustering of pregnancy outcomes within herd. The outcome variable was the total count of non-pregnant females. The respective offsets were the log of the total number of cows and heifers pregnancy tested. The difference in the risk of non-pregnancy between heifers and cows was assessed in this model and all subsequent models were stratified for heifers and cows.

As they could be potential confounders, other management risk factors considered in the models included: if the herd was exposed to community pasture during the summer of 2014; if the herd had more than 300 cows at calving in 2014; the month of first bull exposure in 2014; whether calving started before March 2014; if the calving season was more than 4 cycles or 84 d; and whether or not the herd sold at least some purebred cattle. Models were built using manual backwards stepwise removal. Only factors that were significantly associated with the risk of non-pregnancy or were not significant but were important confounders were retained in the final model. Factors that changed the regression coefficient for infection status by more than 20% when added or removed from the model were considered important confounders. Biologically plausible 2-way interactions were assessed among variables retained in the final model. The difference in non-pregnancy risk between test-positive and test-negative herds was reported as a risk ratio (RR) with 95% confidence intervals (95% CI).

Apparent prevalence was reported with 95% CI (13) for both the proportion of test-positive bulls and positive herds. The true individual prevalence of infection with 95% CI was simulated from

Table III. Distribution of simulation results for true prevalence *Cfv* and *T. foetus* summarizing individual bull PCR results on direct preputial samples (*n* = 735 bulls, 78 herds)

	Median	2.5% percentile	97.5% percentile
Cfv individual bulls			
True prevalence	< 0.01%	< 0.01%	< 0.01%
T. foetus individual bulls			
True prevalence	< 0.01%	< 0.01%	0.38%

* Simulations (n = 20 000) assume a binomial distribution for sensitivity (*Cfv* α = 223, β = 39; *T. foetus* α = 110, β = 13) and specificity (*Cfv* α = 256, β = 46; *T. foetus* α = 338, β = 1). Sensitivity was reported as 85.4% [95% confidence interval (CI), 80.6 to 89.2; 222/260] for *Cfv* (5) and as 90.1% (95% CI: 83.5 to 94.2; 109/121) for *T. foetus* (6). Specificity was reported as 85.0% (95% CI: 80.5 to 88.6; 255/300) for *Cfv* (5) and as 100% (95% CI: 98.9 to 100; 337/337) for *T. foetus* (4).

the apparent prevalence and sensitivity and specificity estimates reported from original validation studies (4–6) using publicly available software (13). Results were reported for 20 000 iterations. The parameters for the beta distribution for the individual animal results were as follows: alpha = x + 1 and beta = n − x + 1, where there were x successes out of "*n*" individuals examined in the original test validation study.

For the herds that had positive test results, the probability of getting the observed number of positive tests if the herd was free of infection was determined using publicly available software (13). The calculations considered the limitations of the sensitivity and specificity of the test, as well as the total number of bulls tested.

Herd level sensitivity and specificity were calculated based on the median and 95th percentile for the number of bulls tested per herd. To estimate herd level sensitivity and specificity, 2 positive reactors for *Cfv* and 1 positive reactor for *T. foetus* were required, assuming a design prevalence of 0.1 and binomial distribution (13). These herd level sensitivities and specificities were then used to estimate the range for true herd prevalence (13).

Finally, the distribution of expected positive and negative predictive values (post-test probabilities of infection with a positive test or freedom from infection with a negative test) were simulated for a range of potential pre-test probabilities of infection using MCMC software (ModelRisk Industrial Version 5.2; Vose Software, Sint-Amandsberg, Belgium) based on reported sensitivity and specificity estimates (n = 10 000 iterations) (4–6,14).

Results

Study population

A group of 105 producers who were recruited to the surveillance network and completed the first survey were invited to participate. A total of 78 producers provided samples from 735 bulls between March 27, 2015 and July 16, 2015. Characteristics of the sample population are provided in Table I. The median number of bulls tested per herd was 7 [interquartile range (IQR): 5 to 12, 95th percentile 24] and the number of bulls tested per herd ranged from 1 to 37.

Pregnancy testing results in fall 2014 and associated herd management factors

Of the 78 producers who submitted samples for testing, 72 (92%) also responded to either a paper or electronic survey about outcomes of pregnancy testing in fall 2014 (Table II). Complete pregnancy test results were available for 57 herds. Most producers who only checked a portion of their herd reported that they did not check cows that were going to be culled for age, temperament, or health reasons. Heifers were more likely to be open than cows (RR: 1.72, 95% CI: 1.19 to 2.50, P = 0.004) and stratification for heifers and cows was included in all subsequent models. None of the other herd management risk factors examined was significantly associated with the risk of non-pregnancy (Table II).

Laboratory results in spring 2015

Ten bulls from 3 herds (Herds A, B, and C) tested positive for *Cfv* or suspect test positive for *T. foetus*. Eight bulls from 2 herds (5 bulls from Herd A and 3 bulls from Herd B) tested positive at least once for *Cfv*. One bull from Herd A and 1 from Herd C was suspect test positive for *T. foetus*. No bulls tested positive for *T. foetus*.

In Herd A, 1 sample was reported as *T. foetus* suspect from the 37 samples submitted on May 7, 2015. This bull was retested and was again suspect on May 29, 2015. One bull was also *Cfv* positive on the first submission of samples from 37 bulls on May 7, 2015. Four additional bulls were test positives by both labs from the 37 samples in the repeat submission from the same herd on May 29, 2015. The age of the *T. foetus* suspect was not available, but 3 of the *Cfv*-positive bulls were 2 y old and 2 were 3 y old. It was reported that 29.4% of the cows and 27.1% of the heifers in Herd A were not pregnant in the fall of 2014. The herd owner reported that 36 of 37 bulls tested had not been exposed to communal grazing. This question was not answered for the final bull, although this bull did not have either a positive or suspect test result. All sampled bulls from this herd were semen tested in the spring of 2015.

In Herd B, 1 of 23 bulls initially examined was test positive for *Cfv* on June 11, 2015. This bull and 1 other also tested positive when the same samples were submitted to a commercial laboratory (PDS). From a second submission of 11 bulls, a third bull was *Cfv* test positive on June 29, 2015. Of the 3 bulls that tested positive at least once for *Cfv*, 1 bull was 2 y old and 1 bull was 4 y old; the age of the final bull was not reported. In Herd B, an open rate of 8.9% was reported for cows and 13.6% for heifers in the fall of 2014. One bull had been exposed to a community pasture, but tested negative for both *Cfv* and *T. foetus*. All sampled bulls from this herd were semen tested in the spring of 2015.

In Herd C, 1 bull of 11 was identified as a suspect positive for *T. foetus* on April 20, 2015. The age of the positive bull was not reported. Herd C did not report pregnancy test data for the fall of 2014, but had indicated on the initial submission that there were no problems with pregnancy rates in the fall of 2014. None of the bulls had been exposed to a community pasture and all sampled bulls from this herd were semen tested in the spring of 2015.

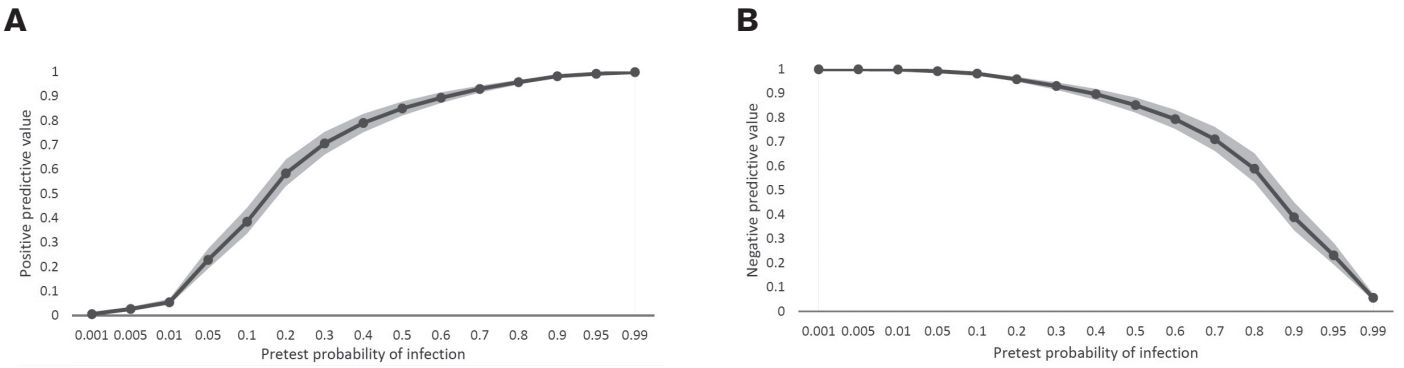

Figure 1. A — Summary of positive predictive values for a range of pre-test probabilities of infection for *Campylobacter fetus* subsp. *venerealis* with 95% confidence intervals. B — Summary of negative predictive values for a range of pre-test probabilities of infection for *Campylobacter fetus* subsp. *venerealis* with 95% confidence intervals.

*** Simulations (n = 10 000) assume a binomial distribution for sensitivity (*Cfv* α = 223, β = 39) and specificity (*Cfv* α = 256, β = 46) (5). Sensitivity was reported as 85.4% (95% CI: 80.6% to 89.2%; 222/260) for *Cfv* (5). Specificity was reported as 85.0% (95% CI: 80.5% to 88.6%; 255/300) for *Cfv* (5).**

Individual bull and herd-level prevalence

The crude apparent individual bull prevalence for *Cfv* was 1.1% (95% CI: 0.5% to 2.1%; 8/735) and apparent herd-level prevalence for *Cfv* was 2.6% (95% CI: 0.3% to 9.0%; 2/78). The crude apparent bull prevalence for *T. foetus* was < 0.001% (95% CI: 0.0% to 0.5%; 0/735) and apparent herd-level prevalence for *T. foetus* was < 0.001% (95% CI: 0.0% to 4.6%; 0/78).

The estimated true prevalence of both *Cfv* and *T. foetus* for the individual bull test results are summarized in Table I, taking into account previously reported clinical sensitivities and specificities of the respective tests for these laboratories and the uncertainty around these estimates associated with sample sizes.

Given the reported specificity of the *Cfv* test, the probability of getting 5 or more false positives from 37 samples collected from Herd A was 0.67. Given the specificity of the *Cfv* test, the probability of getting 3 or more false positive results from 22 samples collected from Herd B was 0.66.

The estimated herd-level sensitivity for *Cfv* when testing 7 bulls (median number tested) and requiring 2 positive reactors was 0.48 and the specificity was 0.72. The estimated herd-level sensitivity for *Cfv* when testing 24 bulls (95th percentile of bulls tested) and requiring 2 positive reactors was 0.98 and the specificity was 0.11. The estimated true herd-level prevalence for *Cfv* was 0.0% (95% CI: 0.0% to 7.3%), assuming a median of 7 bulls tested per herd.

In contrast, the estimated herd-level sensitivities for *T. foetus* when testing 7 and then 24 bulls and requiring 1 reactor were 0.48 and 0.90 and the specificities were 0.999 and 0.999. Based on the median number of 7 bulls tested per herd, the estimated true herd-level prevalence for *T. foetus* was 0.0% (95% CI: 0.0% to 9.8%).

Given the previously reported specificity for the *Cfv* PCR, the expected number of false positive results for 735 samples was 112 (95% CI: 83 to 143). However, only 8 bulls were reported as test positive for *Cfv* in this study. If the previously reported specificity was valid for this group of samples, the probability of getting 8 or fewer false positives was < 0.0001.

Association between laboratory results and the risk of non-pregnancy in fall 2014

For the 57 herds in which pregnancy test results were reported for all cows or heifers, the association between *Cfv* test results for the bulls and the risk of non-pregnancy was modified by the difference between cows and heifers ($P < 0.001$). Cows from herds where at least 1 bull was test positive for *Cfv* were 2.35 times more likely (95% CI: 1.01 to 5.48, $P = 0.047$) to not be pregnant than cows from herds where no positive bulls were identified. However, there was no significant increase in the risk of non-pregnancy for heifers (RR: 1.41, 95% CI: 0.77 to 2.58, $P = 0.27$) in herds where at least 1 bull was test positive for *Cfv*. There were no other significant predictors or important confounders in the final model.

Also related to the significant interaction ($P < 0.001$) between age group and positive *Cfv* tests for any of the bulls in the final model was whether there was a difference between cows and heifers in herds with and without *Cfv* positive bulls. Heifers were significantly more likely to be non-pregnant than cows in herds with no bulls that were *Cfv* positive (RR: 1.78, 95% CI: 1.20 to 2.65, $P = 0.004$). In herds with *Cfv*-positive bulls, however, there was no difference between cows and heifers (RR: 1.41, 95% CI: 0.77 to 2.58, $P = 0.27$).

The association between *T. foetus* status and risk of non-pregnancy was not considered because test results were suspect only and not confirmed as positive in any herd. Follow-up data on pregnancy testing outcomes from the fall of 2015 were not available for the 3 herds with either *Cfv* test positive or *T. foetus* suspect bulls in the spring of 2015.

Estimation of positive and negative predictive values for different pre-test probabilities of disease

Using previously published estimates of clinical sensitivity and specificity (4–6), the post-test probability of infection or positive predictive value of the direct qPCR for *Cfv* was < 90% when the pre-test probability of infection was ≤ 60% (Figure 1A) and < 40% when

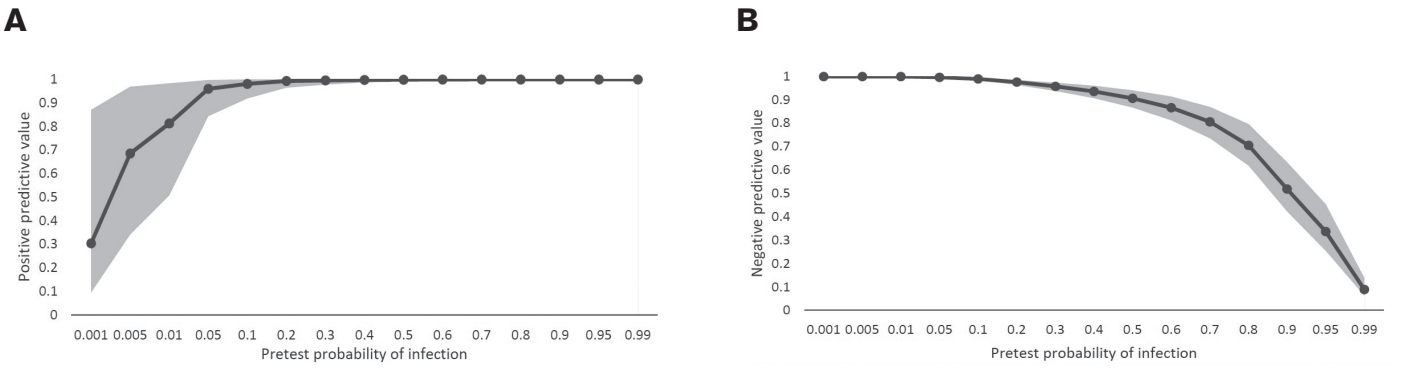

Figure 2. A — Summary of positive predictive values for a range of pre-test probabilities of infection for *Tritrichomonas foetus* with 95% confidence intervals. B — Summary of negative predictive values for a range of pre-test probabilities of infection for *Tritrichomonas foetus* with 95% confidence intervals.

*** Simulations (n = 10 000) assume a binomial distribution for sensitivity (*T. foetus* α = 110, β = 13) (6) and specificity (*T. foetus* α = 338, β = 1) (4). Sensitivity was reported as 90.1% (95% CI: 83.5% to 94.2%; 109/121) for *T. foetus* (6). Specificity was reported as 100% (95% CI: 98.9% to 100%; 337/337) for *T. foetus* (4).**

the pre-test probability of infection was ≤ 10%. When the pre-test probability of infection with *Cfv* was > 80%, the positive predictive value of the test was > 95% and for a pre-test probability > 95%, the positive predictive value was > 99% (Figure 1A). In contrast, when the pre-test probability of infection with *T. foetus* was ≥ 5%, the positive predictive value of the test was > 95% and for a pre-test probability > 20%, the positive predictive value was > 99% (Figure 2A).

The negative predictive values for a single direct qPCR test for the *Cfv* was > 95% when the pretest probability of disease was < 20% and > 99% when the pretest probability of disease was < 5% (Figure 1B). Similarly, the negative predictive values for a single direct qPCR test for the *T. foetus* was > 95% when the pretest probability of disease was < 30% and > 99% when the pretest probability of disease was < 5% (Figure 2B).

Discussion

The results of this study suggest that infections with *Cfv* and *T. foetus* were rare among breeding bulls from a sentinel cohort of cow-calf herds in western Canada. No bulls were positive for *T. foetus* infection and there were only 2 suspects. Given the relatively small number of herds sampled in this cohort and the imperfect nature of the tests, however, and based on the upper bound of the 95% CI, the true individual bull prevalence of *T. foetus* was less than 0.3% and the herd-level prevalence was less than 9.4%. The true prevalence adjusts for validity of the chosen test and is consistent with recent reports from Colorado and Nebraska, areas of the US with comparable management (8). While the sensitivity of the *T. foetus* test reported here was based on direct PCR of preputial samples collected into PBS (6), the specificity used in calculations may not be completely valid for PBS as it was based on samples collected into commercial sampling media (4).

The *Cfv* results were more difficult to interpret. While true individual bull and herd-level prevalence of *Cfv* was estimated to be less than 0.001 based on the previously published sensitivity and specificity of the test, 2 of 78 herds had a total of 8 test-positive bulls. There was also a decreased probability that cows from the herds with an infected bull were pregnant in the breeding season immediately before the test date. This significant association with pregnancy outcomes suggests that the test results might not be false positives as suggested by calculations of true prevalence based on reported test specificity and sensitivity.

The association between qPCR results from direct preputial samples and pregnancy outcomes is in direct contrast to the results of a previous study from New Zealand (15). The New Zealand study identified 29% of 222 tested bulls from 30 beef breeding farms as positive using a similar testing strategy. The authors attributed the lack of a significant association between test results and pregnancy outcomes to a lack of primer specificity. The authors cited previous reports from other regions noting the presence of the parA gene on mobile genetic elements and isolation of *Campylobacter hyointestinalis* from a bull that had tested positive through direct PCR (16–18). They also note that *Cfv* had not been successfully cultured in New Zealand since 1993 (15).

The present study differs from the New Zealand study (15) in that *Cfv* has been cultured from herd outbreaks in western Canada (1) and cultures of clinical isolates have been further verified using whole genome sequencing (Waldner, unpublished data). One of the limitations of the present study compared to the earlier New Zealand study was that there was not enough detail about herd management to directly link bull test results to pregnancy results on individual breeding pastures.

While a study of virgin bulls previously reported that the specificity of the qPCR on direct preputial samples was 85% (5), the current results suggest that the published specificity for adult bulls in western Canada is probably underestimated. There were 8 test-positive *Cfv* results in the present study. Even if we assume that they were all false positives, the probability of having observed 8 or fewer test-positive results was less than 0.0001. The number of observed test positives is far too low to be consistent with the previously reported specificity.

Other evidence that the specificity of the primers used in this qPCR is probably higher for mature bulls in western Canada than previously reported comes from the regional commercial laboratory. Prairie Diagnostic Services reported only 9 positive samples from 468 tested from June 2, 2014 to May 12, 2015 (19). These observations are consistent with previous reports that the effectiveness of

PCR primers for differentiating between subspecies of *C. fetus* varies among regions (20).

As there were no herds that were test positive for *T. foetus* and only 2 herds that were test positive for *Cfv*, there wasn't sufficient power in this sample to look at risk factors for positive herd status. A recent survey of 863 participants in Wyoming identified grazing on public allotments and commingling with other herds as risk factors for *T. foetus* infection (21). In the present study, community pasture exposure was not reported for the test-positive bulls, although 1 herd had at least 1 other bull that had been exposed to communal grazing. There was also no association between community pasture exposure and an increased risk of non-pregnancy in this sentinel cohort.

In another previous study of factors associated with pregnancy outcomes in western Canada, community pasture exposure was associated with an increased risk of non-pregnancy, but only for herds that had not been vaccinated for bovine viral diarrhea virus (BVDV) and infectious bovine rhinotracheitis (IBR). That suggested a higher risk of exposure to infectious disease associated with mixing with other herds in community pastures (22). In the present study, the low prevalence of infection and lack of association between community pasture exposure and pregnancy could be in part attributed to the relatively low frequency of community pasture exposure. Exposure to communal grazing or potential for exposure to bulls or cows from other herds was reported for 10% of bulls from 28% of herds.

Increasing age has also been associated with *T. foetus* infection (7). In the present study, age was not reported for the 2 bulls with suspect *T. foetus* test results, although the bulls that were test positive for *Cfv* ranged in age from 2 to 4 y.

The final objective of this study was to compare the predictive values of positive and negative test results for both *T. foetus* and *Cfv* when screening bulls in the absence of a recognized problem with reproductive performance (low pre-test probability) and when examining bulls as part of the diagnosis of poor pregnancy rates (high pre-test probability). The probability that a positive test result from the direct PCR test indicates *T. foetus* infection is greater than 90% for pre-test probabilities as low as 5%. The positive predictive value drops off quickly, however, as the prevalence of infection in the target population dips below 1%. Similarly, the negative predictive value is greater than 90% until the pre-test probability of infection exceeds 50%. Taken together, the direct PCR *T. foetus* for preputial samples collected directly into PBS can be used for screening low- and moderate-risk bulls with an acceptable probability of false-positive results. However, the probability of false positive becomes very high for very low risk situations such as screening virgin yearling bulls with no history of exposure to an infected herd.

The direct PCR test for *T. foetus* also has high post-test probability for negative results, but due to the imperfect sensitivity, the risk of false negatives from a single test is very high when clinical suspicion of *T. foetus* is above 50%. The sensitivity of the test based on a single sample is less than 100% even when PCR is used with commercial media (4). *Tritrichomonas foetus* is not always recovered from infected bulls during sampling due to the small number of organisms, inconsistent presence, and uneven distribution in the prepuce (7).

This emphasizes the need for 3 repeated tests from at least weekly intervals to rule out infection when clinical suspicion is moderate or high at the time of testing. The 3-sample strategy was recommended in a recent review of *T. foetus* prevention and control regardless of whether culture or PCR was used for testing (8). Based on the simulation results from this study, a single test for *T. foetus* is not appropriate if the pre-test probability of infection is higher than 1%, based on available evidence.

As we are limited to the current reported estimates of specificity of the *Cfv* test, the direct PCR for *Cfv* should not be recommended for routine screening of moderate- and low-risk bulls at this time. The positive predictive value appears to be less than 85% when the pre-test probability of disease is 50% or lower. The costs of false positives that could result from applying the test to low- and very low-risk bulls include the costs of testing, premature culling of valuable bulls, and other potential control measures such as herd vaccination programs. Virgin bulls were excluded from the present study due to the potential for false positives. If positive test results could be confirmed by retesting the bull with culture, the costs of screening moderate-risk bulls might be acceptable. However, most bulls in western Canada are located in areas where the transport time to the lab is greater than 24 h, which compromises the sensitivity of culture (2). As a result, very few herds have access to confirmatory culture with transport times short enough to ensure very good sensitivity, regardless of the use of transport media (9).

The direct PCR test for *Cfv* could potentially be used as a diagnostic test when investigating high-risk bulls from herds with outbreaks of poor reproductive performance consistent with bovine genital campylobacteriosis. To maximize the pre-test probability and resulting interpretation of the test, however, the test is best used after ruling out other common causes of reproductive failure. Because of the limited negative predictive value in high-risk situations, 3 sequential tests have been recommended to provide strong evidence that a bull from an outbreak situation is free of infection and to definitively rule out *Cfv* as the cause of an outbreak (5,10).

The present study reported the results for qPCR applied to preputial scrapings collected directly into PBS. This method has previously been suggested as an opportunity to decrease sampling costs and improve ease of sample handling especially in the winter when there is a high risk of samples freezing in transport (5,6,10). The regional laboratory recommends that *T. foetus* samples should be collected into commercial culture media (In Pouch TF; Biomed Diagnostics) and be incubated at 35°C for 48 h before shipping warm to the laboratory. Use of commercial culture media followed by incubation increases the time and costs associated with sample collection compared to that described in the current study (23). However, the sensitivity of the qPCR for *T. foetus* for a single preputial sample collected into commercial culture media pouches is higher than when collecting directly into PBS, as was done in the present study (6). While this difference is important for a single sample, when 3 samples are collected as recommended, the difference in sensitivity between PBS and the commercial pouches is no longer significant (6).

Additional work is required to identify a more specific and practical field test to screen bulls for *Cfv*. Given the justifiable reluctance of many herd owners to handle their bulls 3 times in succession, improvements to the recommended testing strategy for *T. foetus* would also increase opportunities for disease control. As testing and culling are considered the primary means of managing both *T. foetus* infection and *Cfv* (2), it is important to understand the limitations

and carefully consider how to make the best possible use of the currently available tests while minimizing the risks and costs associated with false positive and negative results.

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